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Among human papillomavirus (HPV) types, clinical association with benign vs malignant lesions correlates with the ability of the corresponding oncogenes to transform cells *in vitro.* However, even though HPV-11 is considered a low-risk type, we have reported previously that the E5a oncogene of HPV-11gt is capable of transforming NIH 3T3 cells in culture. In this study, we found that HPV-11gt E5a and E6 oncogenes have the ability to transform NIH 3T3 and the rat embryo fibroblast line REF 52. Cells were transfected independently with expression plasmids containing the HPV-11gt E5a or E6 oncogenes or both plasmids simultaneously to examine potential interactions. Cells containing these plasmids were phenotypically transformed and had an accelerated doubling time, loss of contact inhibition of growth, and loss of anchorage dependence for cell division. Independent cell lines containing the HPV-11gt E6 gene exhibited variable levels of phenotypic transformation that correlated with the HPV-11gt E6 gene content. The degree of phenotypic transformation could be increased by elevating the level of transcription of the E6 gene, indicating that there is a dose response effect for transformation in this system. These results suggest that increased expression of E6 may be an important factor in malignant progression of naturally occurring tumors.

INTRODUCTION

Human papillomaviruses (HPVs) are transforming DNA viruses that induce cellular proliferation conducive to the development of epithelial tumors (Giri and Danos, 1986; Gissmann *et al.,* 1983; Mounts *et al.,* 1982; Schiffman *et al.,* 1993; zur Hausen, 1987). Although most of these tumors are benign, there is a spectrum of disease that ranges from condylomas or benign warts to premalignant and invasive carcinomas. HPV types that are usually associated with benign lesions, e.g., HPV-6 and -11, are considered low-risk, whereas other types, e.g., HPV-16 and -18, are often referred to as high-risk, since they have been strongly associated with cancer in anogenital and respiratory epithelia (de Villiers, 1989; Gissmann, 1992; Kulski *et al.,* 1990; Lancaster and Jensen, 1978; Wu *et al.,* 1993). It is important to perform studies *in vitro* that allow comparative analyses of high- vs low-risk HPV types as a means to identify differences that may uncover critical elements required for HPV oncogenesis. As it is currently understood, the process of HPV oncogenesis is complex and results primarily from the combined effects of oncoprotein function and levels of expression (Barbosa *et al.,* 1991; Shah and Howley, 1990). Thus, the functional differences between low- and high-risk viruses that infect the same types of epithelia may result from varying oncoprotein potency and the oncoprotein levels attained in those particular cells.

The relative oncogenic potential of different HPV types can be compared *in vitro* by testing the ability of the respective DNAs to immortalize primary cell lines, or alternatively, to transform established rodent and human cells in culture after transfection. The immortalization assay primarily evaluates the role of HPV as an "initiator," whereas the transformation assay evaluates its role as a "promoter" of tumor induction. These types of assays have identified the oncogenes of HPV as E5, E6, and E7 (Barbosa *et al.,* 1991; Chen and Mounts, 1990; Halbert *et al.,* 1992, 1991; Hiraiwa *et al.,* 1993; Kiyono *et al.,* 1992; Storey and Banks, 1993). When studies have compared the relative oncogenic potential among the oncogenes obtained from high- vs low-risk types, the former have usually been stronger *in vitro* (Schmitt *et al.,* 1994). For example, the E7 gene of HPV-16 or -18 is sufficient to immortalize primary human epithelial cells (Halbert *et al.,* 1991), while HPV-6 E7 is not (Halbert *et al.,* 1992). This finding has also been observed in the transformation assay, and the early region genes of high-risk types have been able to cooperate with other oncogenes, such as activated *ras,* in the transformation of rodent cells, while early regions of low-risk types have not (Storey and Banks, 1993). In a transformation assay of human embryonic fibroblasts with HPV-16 early genes, both E6 and E7 were found to be necessary (Storey *et al.,* 1990). Studies have shown that a limited transforming activity can be measured for the early regions of HPV-6 and HPV-

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11 if expression is regulated by heterologous promoters (Storey *et al.,* 1988). In our laboratory, the transforming activity of the E5a gene from an independent isolate of HPV-11 (HPV-11gt) has also been identified using NIH 3T3 and C127 cells (Chen and Mounts, 1990). We are interested in studying the transforming potential of oncoproteins from HPV-11 as well as possible interactions between them in order to identify differences relative to high-risk types that may help with our understanding of HPV oncogenesis.

The E6 gene encodes a protein that is approximately 150 amino acids long. This protein contains four Cys-X-X-Cys repeats, which are highly conserved in position and number throughout the different oncogenic types and which are characteristic of the zinc finger domains of some DNA binding proteins (Berg, 1986). HPV-18 E6 binds to zinc *in vitro* (Grossman and Laimins, 1989) and HPV-16 E6 has transcriptional trans-activating capabilities (Sedman *et al.,* 1991). Another function of E6 is the binding of the tumor-suppressor protein p53 in a complex that results in the accelerated degradation of this factor through the ubiquitin-dependent proteolysis system (Crook *et al.,* 1991). The association of E6 with p53 is mediated by an additional cellular factor designated E6-AP, for E6-associated protein (Huibregtse *et al.,* 1991). However, the ability of the E6 proteins of viruses of different oncogenic potential to bind to p53 seems to vary (Werness *et al.,* 1990; Farthing and Vousden, 1994).

In this study, we report that the HPV-11gt E6 gene is able to transform NIH 3T3 and REF 52 cells (rat embryo fibroblasts) in culture. The transfected cells presented varying degrees of transformation. We determined the E6 gene content in cells with different phenotypes, and we also investigated the effect of increasing transcription of this gene by making use of an inducible promoter, the mouse metallothionein I promoter. Our results are consistent with the hypothesis that the degree of phenotypic transformation of cells transfected with the HPV-11gt E6 oncogene is related to E6 expression determined by the copy number of the E6 gene and by the level of transcription.

MATERIALS AND METHODS

Plasmid constructions

The expression vector pMT.neo.1 (Peden *et al.,* 1989) was employed for cloning the HPV-11gt E6 gene. This recombinant vector carries resistance to the aminoglycoside antibiotic G418 under the control of the herpesvirus thymidine kinase promoter and allows selection of eukaryotic transfectants. The vector also confers resistance to ampicillin for selection in bacteria. In addition, this vector contains the mouse metallothionein type I promoter immediately upstream of the cloning site. This promoter offers the advantage that it may be induced to increase the levels of transcription with heavy metals such as zinc (Peden *et al.,* 1989). The plasmid pMT.neo.1 contains the SV40 splice and polyadenylation signals downstream of the cloning site.

Polymerase chain reaction (PCR) amplification

To construct the plasmid pMT-E6.neo, the HPV-11gt E6 was first amplified with ampliTaq DNA polymerase (Perkin–Elmer–Cetus, Norwalk, CT) using the plasmid pHP-11gt containing the entire HPV11gt genome (Metcalfe *et al.,* 1989) as the template. The 5′ end primer 5′-ACGAAGCTTTATGGAAAGTAAAGATGCCT-3′ and the 3′ end primer 5′-AGTTCTAGATCCTTTAGGGTAACAAGTCT-3′ contain base mismatches (underlined) in positions 5, 8 and 4, 8, respectively. These mutations were introduced to generate a *Hin*dIII site in the 5′ end primer and a *Xba*I site in the 3′ end primer as highlighted in bold. The resulting 476-bp product containing the 453-bp E6 gene plus the restriction sites was then digested with *Hin*dIII and *Xba*I for cloning. The same strategy was used to clone the E6 PCR product into the vector pGEM 2 (Promega, Madison WI), which contains flanking promoters for T7 or SP6 RNA polymerases, and into pBluescript II SK (Stratagene, La Jolla, CA), which contains promoters for T3 and T7 RNA polymerases. Successful cloning was detected by analysis of restriction enzyme digests and was verified through DNA sequencing (Sanger and Coulson, 1975), using a commercially available sequencing kit (USBC, Cleveland, OH). Oligonucleotides used as primers were synthesized by the Johns Hopkins Biopolymers Laboratory.

Cell culture and DNA transfection

NIH 3T3 cells were kept as monolayer cultures in Eagle Minimal Essential Medium (MEM; GIBCO Laboratories, Grand Island, N.Y.), with 10% calf serum (Hyclone, Logan, UT). REF 52 clone 6 cells (Logan *et al.,* 1981) were obtained from Keith Peden (CBER, FDA) and were grown as monolayer cultures in MEM, but were supplemented with 5% fetal bovine serum (Hyclone). NIH 3T3 cells were transfected with 2 μg of plasmid DNA using the Transfinity calcium phosphate precipitation kit (BRL Laboratories; Gaithersburg, MD), selected in medium containing G418, and scored for transformation as published (Chen and Mounts, 1990). REF 52 cells were transfected with 3 μg of DNA using the Lipofectamine reagent (BRL Laboratories). The plasmid pMT-E5ab.neo (Chen and Mounts, 1990) was used as a positive control for transformation.

Establishment of cell lines

Individual G418-resistant ($G418^r$) colonies were picked using a sterile cotton applicator pre-wet with a solution containing 0.1% Trypsin in PBS and expanded in 24-well plates.

Growth curve and anchorage independence assay

The doubling times, saturation densities, and colony formation in suspension were determined as described

previously (Chen and Mounts, 1990), except that 2 to 4 $\times$ 10^3 cells were plated in semisolid medium. Experiments using induction with zinc were performed at 25, 50, and 75 μM zinc sulfate concentrations in the medium.

Statistical analysis

For comparisons between means, a double-tailed Student *t* test was employed (Sort, 1990). For comparisons between percentage growth in semisolid medium, the difference between the cells that were transfected with HPV sequences versus the control was tested using the Kruskal–Wallis test, H = 30.631, DF = 5 (Sort, 1990). Comparisons among all the percentages were done using the Boferroni-adjusted Mann–Whitney, $d = 0.10/15 = 0.0067$ (Sort, 1990).

Southern hybridization

Total cellular DNA was extracted from the cells as published (Mounts and Kelly, 1984). Twenty micrograms of DNA per sample were digested with *Eco*RI and size fractionated by 1% agarose electrophoresis and transferred onto nitrocellulose filters, 0.45 μm pore size (Schleicher & Schuell, Keene, NH) as described previously (Mounts and Kelly, 1984). Hybridization was performed using a riboprobe labeled with [α-^{32}P]CTP by *in vitro* transcription with SP6 RNA polymerase (Promega) using the plasmid pGem2.HPV-11gtE6 as template. Filters were washed at high stringency conditions. Autoradiographs were obtained using Kodak XAR X-ray film (Eastman Kodak, Rochester, NY) with intensification at $-70°$.

Northern hybridization

Cells were plated in medium without additional zinc. Twenty-four hours later, zinc sulfate was added to half of the dishes to achieve a final concentration of 50 μM and grown for 24 hr more. Total RNA was extracted by the guanidinium thiocyanate-cesium chloride centrifugation method for Northern analysis as published previously (Ward and Mounts, 1988). Purification of poly(A)+ RNA from total RNA was done using an Oligotex Direct kit (Qiagen, Chatsworth, CA).

TABLE 1

Drug Resistance and Focus Formation of Transfected NIH 3T3 Cells

Plasmid transfected	Number of G418-resistant colonies		Number of transformed foci (%)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Mock	0	0	0 (0)	0 (0)
pMT.neo. 1	68	22	10 (15)	1 (4.5)
pMT-E6.neo	68	12	41 (60)	6 (50)
pMT-E5ab.neo	39	52	33 (85)	22 (42)
pMT-E6.neo + pMT-E5ab.neo	143	61	126 (88)	37 (61)

TABLE 2

Drug Resistance and Focus Formation of Transfected REF 52 Cells

Plasmid transfected	Number of G418-resistant colonies	Number of transformed foci (%)
Mock	0	0 (0)
pMT.neo.1	185	15 (8)
pMT-E6.neo	299	163 (55)
pMT-E5ab.neo	259	146 (56)

RESULTS

Sequence of HPV-11 E6

The nucleotide sequence obtained for the E6 gene from the HPV-11gt genome that was molecularly cloned from a *condyloma acuminatum* of the cervix (Metcalfe *et al.,* 1989) revealed an identical length to the prototype HPV-11 (Dartmann *et al.,* 1986). Although four base changes were identified, A to G, T to C, C to T, and T to C at positions 132, 137, 380, and 402 relative to the prototype, only the first one resulted in a change at position 12, from a threonine to an alanine, in the 151 amino acids.

Focus forming activity of E6 in NIH 3T3 and REF-52 cells

Foci were scored by microscopic examination on the basis of morphological transformation, which included rounding, smaller size, higher refringency, and growth in multiple layers. NIH 3T3 cells transfected with E5a-b were able to form foci in a high percentage of the colonies, as published (Chen and Mounts, 1990), and served as our positive control for transformation. Cells that were transfected with E6 were also able to form foci as presented in Table 1. In two independent experiments, NIH 3T3 cells that were transfected with pMT-E6.neo grew transformed colonies in a higher percentage (85 and 42%) than cells that were transfected with the pMT.neo.1 vector alone (15 and 4.5%). There were more transformed colonies when plasmids containing E5a and E6 were transfected in combination. As expected, no G418^r colonies were obtained from mock transfection without pMT.neo.1 plasmid DNA. Similar results were obtained in a different cell line, rat embryo fibroblasts, REF 52, in which there was also a higher percentage formation of transformed colonies in cells transfected with pMT-E6.neo (55%) or pMT-E5ab.neo (51%) than in cells transfected with pMT.neo.1 (8%) (Table 2).

Phenotype of NIH 3T3 cells transfected with HPV-11 genes

Individual G418^r colonies were isolated as described under Materials and Methods, and some of the resulting

TABLE 3

Transformed Phenotype of NIH 3T3 Cells Transfected with E6 and E5a-b ORFs

Plasmid transfected	Cell line	Population doubling time (hours)		Saturation density (Cells/cm^2)	
		10% serum	2% serum	10% serum	2% serum
pMt.neo.1	N.neo.A6	50	149	1.8×10^4	7.0×10^3
pMT-E6.neo	N.E6.B4	28	86	8.9×10^4	2.5×10^4
	N.E6.B5	20	57	1.8×10^5	1.8×10^4
pMT-E5ab.neo	N.E5.C5	22	96	5.4×10^4	2.1×10^4

lines were further characterized by determining the population doubling time and saturation density in complete growth medium as well as in medium reduced in serum (Table 3). The population doubling time was reduced in the cell lines that were transfected with plasmids containing the HPV-11gt genes as compared with a cell line that was transfected with the vector alone. In complete medium supplemented with 10% calf serum, two cell lines containing the HPV-11gt E6 gene, N.E6.B4 and N.E6.B5, had almost half the population doubling time of N.neo.A6, a cell line that contained the vector sequences alone. These differences were still apparent when the growth curves were performed in medium with reduced serum concentration. The E5a-b-containing cell line, N.E5.C5, was comparable to the E6-containing lines in its growth rate. Similarly, cell lines expressing E5a-b and E6 were able to grow to a higher density than N.neo.A6 even when grown in reduced serum concentration. Of the two cell lines containing the HPV-11gt E6 gene, it seems that N.E6.B5 grows faster than N.E6.B4. It is also apparent that it achieves a higher saturation density than N.E6.B4 in complete medium, but not in medium with a reduced concentration of serum.

The ability of these lines to achieve anchorage-independent growth was examined by culture in soft agarose (Table 4). Cells containing plasmids with the E5a-b and E6 genes, alone or in combination, were able to form colonies in suspension and are therefore considered transformed under this assay. In contrast, cells containing the vector alone were not able to form colonies in suspension. The manifestations of transformation obtained in two cell lines that were transfected with both E5a-b and E6 were not significantly different than those obtained for E5a-b or E6 alone. As it had been apparent when we measured the doubling times and saturation densities for N.E6.B4 and N.E6.B5, there was also a difference in the level of transformation obtained in these cell lines in the anchorage-independence assay. Once again, N.E6.B5 was more transformed with 57.4% of the cells forming colonies as compared with 4.5% for N.E6.B4. The phenotypic differences observed between independent cell lines that contain the plasmid pMT-E6.neo led us to hypothesize that these might result from a difference in the genetic load of pMT-E6.neo, i.e., the number of copies of this plasmid in each line.

TABLE 4

Anchorage Independence Assay for NIH 3T3 Cells Transfected with E6 or E5a-b ORFs

Plasmid transfected	Cell line	Percentage colonies Mean* (± SE, *n*)
pMT.neo.1	N.neo.A6	0.0^a (± 0.0, 7)
pMT-E6.neo	N.E6.B4	4.5^b (± 0.6, 4)
	N.E6.B5	57.4^c (± 2.6, 8)
pMT-E5ab.neo	N.E5.C5	76.2^d (± 5.1, 12)
pMT-E5ab.neo + pMT-E6.neo	N.E5E6.A2	73.0^d (± 1.5, 8)
	N.E5E6.D3	59.5^c (± 0.9, 4)

Note. *Means identified by different superscript letters are statistically different from other letter designations. SE, standard error; *n*, number of dishes scored.

Southern analysis

Genomic DNA was extracted from some of the transformed cell lines for analysis by Southern hybridization to corroborate the presence of the E5a-b and the E6 genes in the corresponding cells (Fig. 1A). Genomic DNA was digested with *Eco*RI, since this would release the E6 gene contained within a 3.3-kb fragment of the plasmid pMT-E6.neo and would allow comparison of the relative amounts of this gene in the two E6-transformed cell lines. For the standards, genomic DNA was extracted from untransfected NIH 3T3 cells and mixed with different amounts of the pMT-E6.neo plasmid and digested in parallel with *Eco*RI. The E6 riboprobe hybridized specifically to the expected 3.3-kb band in the standards as shown in lanes 6–8. The corresponding band was visible in lanes containing DNA from the E6-containing cell lines N.E6.B5 and N.E6.B4, lanes 1 and 2, respectively. This band was absent in lanes containing DNA from cell lines N.E5.C5 (lane 3), N.neo.A6 (lane 4), and untransfected NIH 3T3 cells (lane 5). Some background hybridization was seen at a higher molecular weight even in the negative controls. When comparing N.E6.B5 vs N.E6.B4, the E6 gene hybridization signal observed in the autoradiography was approximately 10-fold higher for N.E6.B5, which exhibits a higher level of transformation than N.E6.B4. This indicates a correlation between gene dosage and phenotypic transformation and suggests the hypothesis that higher levels of E6 lead to a more trans-

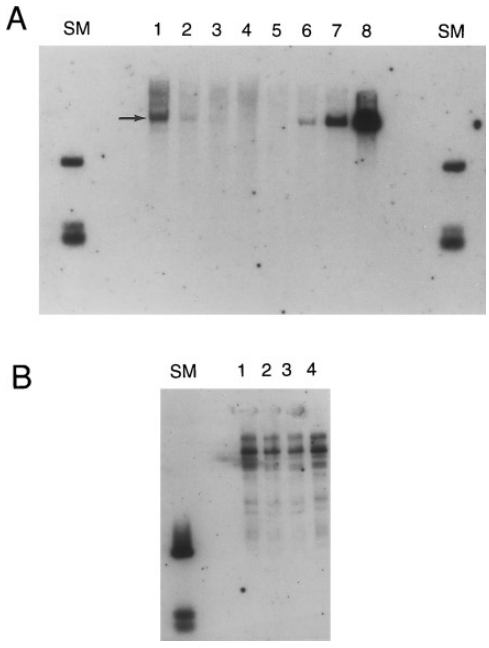

FIG. 1. Detection of HPV-11gt E6 sequences in genomic DNA extracted from transformed cells lines. (A) Twenty micrograms of genomic DNA from cell lines N.E6.B5 (lane 1), N.E6.B4 (lane 2), N.E5.C5 (lane 3), N.neo.A6 (lane 4) were digested with *Eco*RI, size fractionated in a 1% agarose gel, and analyzed by Southern transfer and hybridization with a ^{32}P-labeled riboprobe containing the HPV-11gt E6 sequence. As a negative control, NIH 3T3 genomic DNA was treated in parallel (lane 5). Purified pMT-E6.neo plasmid was added to NIH 3T3 DNA in the amounts of 4 pg (lane 6), 40 pg (lane 7), and 400 pg (lane 8), as standards. The arrow points to the expected 3.3-kb band. Bands in other positions indicate integrations that occurred within the 3.3-kb fragment. A 1-kb ladder (BRL) was used as size markers (SM). (B) A duplicate filter was hybridized with a ^{32}P-labeled riboprobe containing the sequence of the mouse β-actin gene.

formed phenotype. A filter loaded in duplicate for each cell line was hybridized with a riboprobe specific for the mouse β-actin gene (Fig. 1B) and shows that small differences in the amounts of DNA loaded did not account for the differences in hybridization using the E6 riboprobe. The specificity of the DNA present in the cell lines was confirmed by hybridizing a duplicate filter with a riboprobe complementary to the E5a-b genes, where hybridization was only detected in DNA from N.E5.C5 (data not shown).

Induction of the transcription of E6

Since a difference in the transformed phenotype of two cell lines containing different amounts of the E6 gene was apparent, we were interested in testing the hypothesis that this correlated with levels of E6 transcription. Therefore, we examined if increasing the level of E6 would result in increased transformation. Since the E6 gene is located downstream of a metallothionein promoter, the level of transcription can be increased by adding zinc to the medium. Four cell lines that had been transfected with pMT-E6.neo were cultured in soft agarose in the presence or absence of zinc sulfate added to the final concentrations of 25, 50, or 75 μM. There was an increased number and size of colonies in three of four cell lines containing pMT-E6.neo when grown in the presence of zinc (Fig. 2). There were no statistically significant differences among the number of colonies obtained with the three zinc concentrations, and therefore the data were compounded for further analysis. The increase in anchorage-independent growth of cells in the presence of zinc was statistically significant for the cell lines N.E6.B4, N.E6.B6, and N.E6.B7 (Fig. 3). Relative increments in transformation were larger in cells with lower basal transformation than in cells that were already more transformed. For example, N.E6.B7 formed colonies in only 14% of the cells without zinc, and this number was increased to 38.2% when zinc was added to the medium. For N.E6.B4 the increase was from 24% without zinc to 41.8% with zinc, and for N.E6.B6 from 36% without zinc to 45% with zinc. There was no significant difference in percentage growth in N.E6.B5 when cultured with zinc (59.3%) vs without zinc (55.5%). However, this cell line exhibited the highest baseline growth.

Northern analysis

In order to confirm that the addition of zinc to the medium is increasing the levels of transcription of the E6 gene, the level of E6 mRNA in two cell lines with different transformed phenotypes, N.E6.B4 and N.E6.B5, which were grown in the presence or absence of zinc, was examined using Northern hybridization. Ethidium bromide staining of the agarose gel prior to the transfer shows comparable amounts of RNA loaded for each sample (Fig. 4A). The autoradiogram obtained after hybridization with the E6 riboprobe shows the presence of E6 message in RNA from N.E6.B5 cells that had been cultured in the presence of zinc (Fig. 4B, lane 5) but not in uninduced cells (lane 4). Duplicate filters showed the presence of E6 message in uninduced N.E6.B5 only after prolonged exposure (data not shown). The E6 message was not detectable in N.E6.B4 cells (lanes 2 and 3). For these cells, Northern analysis of poly(A)+ RNA confirmed the presence of the E6 message after induction with zinc (Fig. 4C, lane 3), but not in the uninduced state (lane 2). Total and poly(A)+ RNA from N.neo.A6 cells were included as negative controls (lane 1).

DISCUSSION

The comparisons of the relative potency of the proteins of HPVs with different oncogenic potential as well as their relative levels of expression have been important areas in the research of HPV oncogenesis (Barbosa *et al.,* 1991; de Villiers, 1989; Gage *et al.,* 1990; Hiraiwa *et al.,* 1993; Iwasaka *et al.,* 1993; Kiyono *et al.,* 1992). Our study is the first to obtain transformation of NIH 3T3 and REF 52 cells upon transfection with a plasmid encoding the HPV-11gt E6 and it is also the first to show transformation of rat embryo fibroblasts with HPV-11gt E5a-b genes. Other systems using different methodologies and

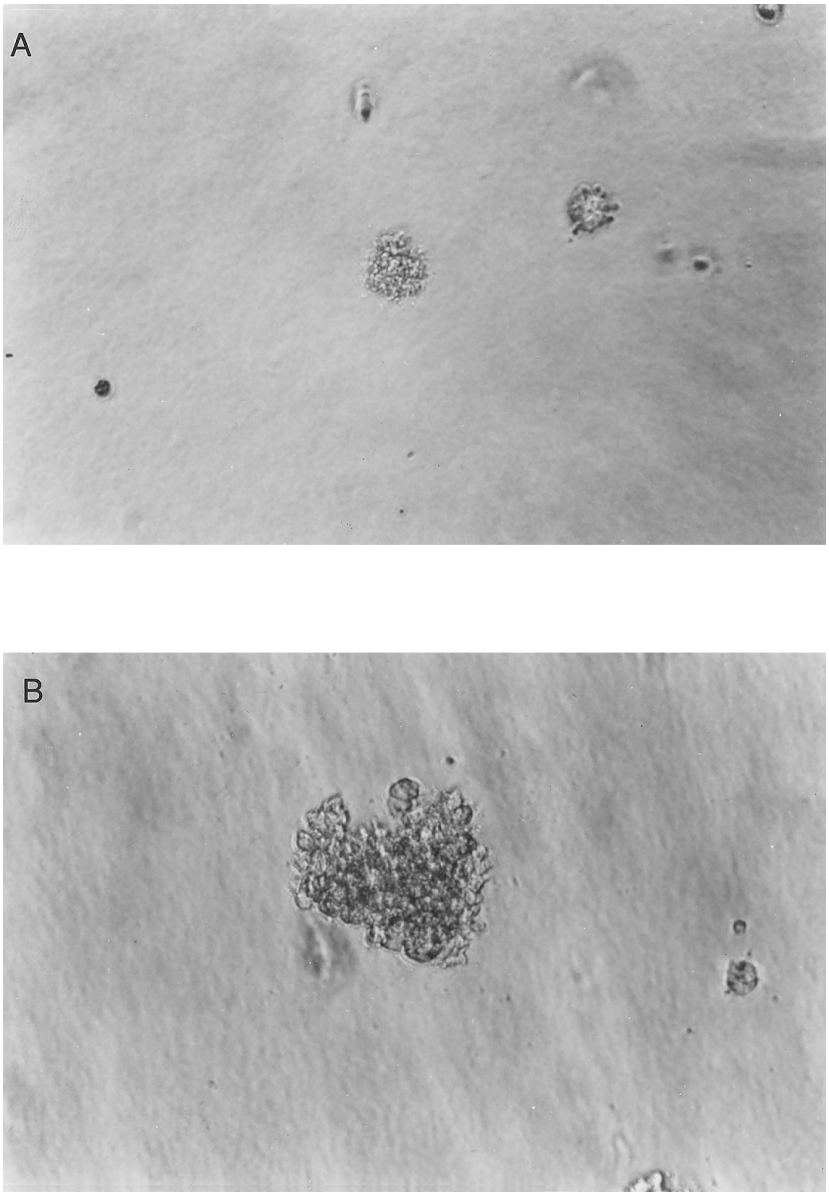

FIG. 2. Increase in anchorage independence of pMT.E6.neo containing NIH 3T3 cells in medium added with zinc. N.E6.B4 cells were plated in MEM medium containing 0.5% agarose (A) vs identical medium with zinc sulfate added to 75 μM (B). Photomicrographs of representative colonies were taken on Day 22 (200×).

cell types have not succeeded in identifying a transforming potential in the early region of HPV-11. In one study, the plasmid employed contained the entire HPV-11 genome placed downstream of a Moloney Murine Leukemia Virus LTR, and it was unable to transform primary rat kidney epithelial cells (BRK) (Storey *et al.,* 1988). Another study did not obtain a transforming activity of HPV-11 E6 in 3Y1 rat fibroblasts (Hiraiwa *et al.,* 1993). Some of these earlier studies have shown an overall low level of colony formation in suspension by pooling the transfected cells and plating them in soft agar medium. Our strategy involved preselecting and establishing individual cell lines with varying morphologies obtained from $G418^r$ colonies. Each line presented variable levels of colony formation in suspension. This improves the scoring of cell lines with a more transformed phenotype by reducing the background of nontransformed colonies present in a more heterogeneous population. The spectrum of transformation presented by different cell lines is probably determined by the number of plasmids that are integrated into the particular genomes and the individual sites of integration.

In other studies, the oncogenic potential of HPV-6b E6 was tested in NIH 3T3 cells and no transformation was obtained (Barbosa *et al.,* 1991). A direct comparison with this experimental system becomes more difficult, since the amino acid sequence of HPV-6b E6 (Schwarz *et al.,* 1983) has thirty substitutions compared to the sequence of HPV-11gt that we used in our experiments. Relative to the prototype HPV-11 E6, which was molecularly cloned from a respiratory tract papilloma (Gissmann *et al.,* 1982), there is only one amino acid change in the

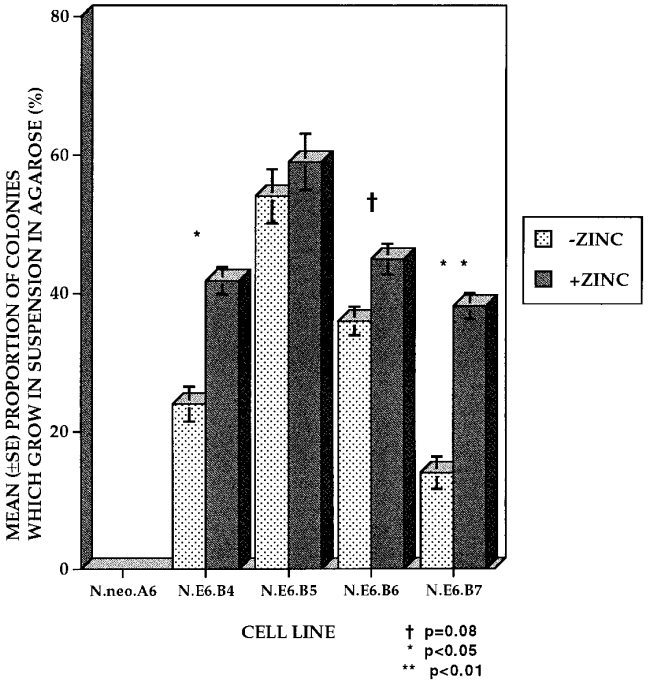

FIG. 3. Relative increments in percentage colony formation in suspension for cell lines containing the plasmid pMT-E6.neo after induction with zinc. The cell line N.neo.A6 was included as a negative control. SE, standard error.

sequence of HPV-11gt E6, which we are studying. The E6 gene in the prototype HPV-11 was not able to complement E7 in the immortalization of human foreskin keratinocytes but was not evaluated for its ability to transform NIH 3T3 cells in these studies (Barbosa *et al.,* 1991).

We have used an inducible promoter and identified a correlation between the levels of transcription of the E6 gene and the transformed phenotype, supporting the hypothesis that transformation by HPV11gt-E6 is dose dependent. Since the increase in transformation upon induction with zinc was lower in cells that were already more transformed than in those that were less transformed, it appears that the transformation process is saturable.

It is presently proposed that one potential mechanism for the oncogenic potential of the E6 gene is exerted through the binding of the tumor suppressor p53, which is mediated by E6-AP (E6 accessory protein) (Huibregtse *et al.,* 1991). This association leads to an accelerated degradation of p53 through the ubiquitin-dependent proteolysis system (Scheffner *et al.,* 1990). Furthermore, the regions within E6 that are responsible for binding are different from those involved in the degradation of p53, and there appears to be a correlation between the oncogenic HPV type and the level of induced degradation of p53 *in vitro* (Crook *et al.,* 1991). In that regard, HPV-11 E6 has been able to bind to p53 *in vitro,* but it does not function in accelerating the degradation of p53 (Crook *et al.,* 1991). The region in E6 involved in p53 degradation has been localized to the N terminal half of the protein, and it is in this half that the amino acid change in HPV-11gt E6 was identified. However, p53-independent alternatives are also being investigated (Sedman *et al.,* 1992; Chen *et al.,* 1995).

It will also be important to test functional interactions of this protein with other HPV proteins such as E7, since in many systems, high-risk type E6 genes but not low-risk types have been able to have a cooperative effect with E7 (Sedman *et al.,* 1991). In our studies, we also examined the phenotype of cells transformed with both HPV-11 E6 and E5a-b and did not find a level of transformation that was significantly higher than that of cells transfected with E5a-b alone. These results suggest that the interaction between these two gene products is not necessary for the induction of a transformed phenotype, but it does not rule out the possibility that an interaction between these two oncogenes expressed jointly at lower levels may have a synergistic effect. To test this hypothesis it would be appropriate to employ cells that are more resistant to oncogenes when transfected individually, such as primary human epithelial cells.

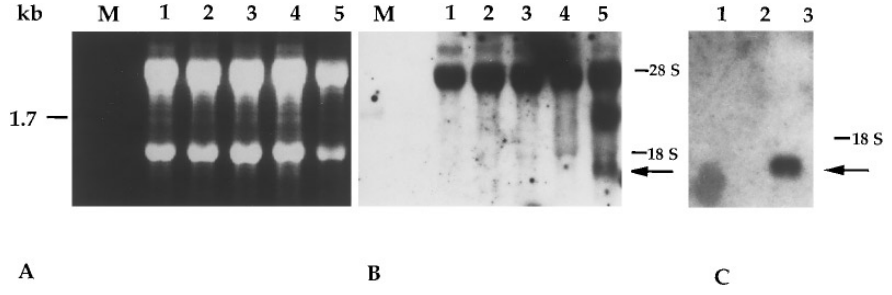

FIG. 4. Analysis of HPV-11gt E6 mRNA in transformed cell lines. Ten micrograms of total RNA were size fractionated in a 1.6% agarose gel containing 2.2 *M* formaldehyde and analyzed by Northern transfer and hybridization with an HPV-11gt E6 antisense riboprobe labeled with ^{32}P. (A) Photograph of the agarose gel stained with ethidium bromide showing 18 S and 28 S ribosomal RNA. N.E6.B4 (lanes 2 and 3) and N.E6.B5 (lanes 4 and 5) were grown in parallel in MEM (lanes 2 and 4) vs medium with zinc added to 50 μM (lanes 3 and 5). N.neo.A6 was included as a negative control (lane 1). A 1-kb ladder (BRL) was used as size markers (M). (B) Autoradiogram obtained after hybridization of the nitrocellulose filter. The arrow points to the predicted position of the E6 message visible in lane 5. There was cross-hybridization of the probe with ribosomal RNA, predominantly 28 S, which served as an internal standard. The radioactive spot in the middle of lane 5 was absent in duplicate filters. (C) Autoradiogram obtained after Northern analysis of poly(A)+ RNA obtained from N.neo.A6 (lane 1) and N.E6.B4 grown in MEM (lane 2) vs medium with zinc (lane 3). The arrow points to the E6 message.

The inducibility of this expression system makes it especially valuable since the effects of over-expression of E6 may be studied *in vivo.* Doebritz *et al.* (1994) found that the transformed phenotype of a cervical carcinoma cell line, SW 756, also correlates with the level of expression of HPV-18 E6 and E7. The demonstration that the phenotypic transformation of cells transfected with the HPV-11gt E6 oncogene is related to the level of E6 expression suggests that the identification of low-risk HPVs in malignant tumors (Byrne *et al.,* 1987; Kulski *et al.,* 1990; McGlennen *et al.,* 1992; Sutton *et al.,* 1987; Wu and Mounts, 1989) may result from an elevated level of E6. Such an elevation in E6 levels may arise by an increase in the copy number of the viral genome through enhanced DNA replication or by an increase in the transcription of E6, and this may be an important factor in malignant progression of naturally occurring tumors.

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